DEDICATION

The staff of the Basic and Applied Research Department dedicate this report to the memory of Paul J. Heberlein, Ph.D. who contributed so much to the conceptualization and prosecution of this work and whose goodness, energy, insight and great potential for science were taken from us by his untimely death on November 24, 1969.

ACKNOWLEDGEMENTS

This work was supported by the Biosciences Office (Code SB) of the Office of Space Sciences and Applications, National Aeronautics and Space Administration. We wish especially to acknowledge the encouragement and advice provided by Dr. Orr Reynolds and Dr. George Jacobs of that organization, without whose help this work could not have been accomplished.

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1.0 OVERVIEW

This section describes briefly the general rationale and direction of the NASw-870 contract effort during the period 1964 through 1970. Subsequent sections will describe in more detail the two major scientific and technical efforts prosecuted under the contract and their justification and accomplishments.*

Since the advent of the earth satellite, the biological sciences community has been interested in the behavior of biological specimens in the space environment. Several bioscientific objectives can be achieved only in the space environment. The physiologic and psychologic behavior of man when exposed to the unique space flight environment is of great interest. Less complex biological specimens can be used to study aspects of basic physiologic functions. Also, there are important basic biological phenomena thought to be related to the interaction of the geosphere with primary physiological functions which can only be studied by removing the specimen from the geosphere. Thus, space flight provides a unique opportunity to better understand the biosphere.

In 1963, planners in NASA's Office of Space Sciences and Applications determined that the question of interaction of geophysical forces and basic biological phenomena might be approached through measurement of oxidative metabolism.

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*Bibliographies, certain drawings, diagrams and photographs, etc., are not cited or reproduced herein but, as necessary, are related by footnotes to the primary project documents submitted earlier.

Oxidative metabolism is an important primary life process, is often an excellent secondary manifestation of other primary processes, and is amenable to measurement.

But no respirometer was then available, satisfying both space flight constraints and scientific data requirements. Accordingly, Space/Defense Corporation undertook the development of such a microrespirometer under Contract NASw-870. The hardware development was organized around the concept of an experiment in biological rhythmicity; the parameter to be measured was the oxidative metabolism of the common potato whose respiratory rhythmicity was hypothesized to be influenced by geophysical forces. The NASw-870 plan, then, called for the development of a life support system and microrespirometer potentially capable of space flight and able to measure the variation in oxygen consumption of a single potato sprout. Furthermore, the plan called for the eventual development of another configuration able to measure the respiration of several specimens individually and/or collectively. This larger configuration would be useful should a flight opportunity occur with more relaxed weight, volume and power requirements where biostatistical data requirements could be more readily satisfied. The program prosecuted to attain these scientific and technical objectives is described in paragraph 2.0, Respirometer Development Program.

In 1966, NASA planning projected the need to develop a bioexperimental system to study certain basic endocrinophysiologic responses to weightlessness. The specific scientific

question was how organisms responded, hypothalamically, to weightlessness. Fish were chosen as the experimental animal. The NASw-870 development plan in 1967, then, added the requirement to: first, develop a fresh water teleost life support system potentially capable of space flight and; second, demonstrate the ability to measure fishes posterior hypothalamic response to altered orientation in the gravitational field. Paragraph 3.0, below, Gravity and Neurohypophyseal Secretory Activity, describes the program prosecuted by Space/Defense toward these technical and scientific objectives.

2.0 RESPIROMETER DEVELOPMENT PROGRAM

2.1 Biological Review and Rationale*

Biorhythmicity in behavioral and physiologic activity has long been recognized. As techniques in disciplined observation were improved, it soon became clear that all living things demonstrated some degree of periodicity and that if enough data were collected about nearly any biologic parameter and proper analytic methods were used, a periodicity appeared to be present. The periods displayed by organisms in nature reflect the principal cyclic event of their immediate environment. Thus, such

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*For a more detailed discussion of the biology and complete bibliographic citation of the scientific arguments, see S/D P69-136, dated 27 May 1969, "Periodicity of Potato Respiration" (supplied in response to NASA letter PBA 176 (c7.2) dated 1 May 1969); and "Design and Development of a Microbiological Respirometer with Space Flight Applications." P.C. Taudvin, B.W. Pince and J.M. Paros, Symposium Minutes, 17th Aerospace Instrumentation Conference, ISA, May 5-6, 1971.

periods as solar day, tidal, synodic month, and annual have been observed in a wide variety of plants and animals.

When organisms are carefully shielded from the rhythmic variations in all ordinary factors of their environment, such as light, temperature, humidity, and tides, their behavior still remains rhythmic. Under such controlled conditions, however, a rhythmic duplicity commonly becomes evident. Precise, mean, environmentally related frequencies (dian, lunar, monthly, annual) remain present, but, in addition, the overt daily or tidal physiological patterns may now display regular periods differing somewhat from the moon- or sun-related ones. In other words, the events in the recurring patterns occur regularly either a little earlier or a little later each day. These odd-lengthened periods have been the reason for coining the term circadian (circa = about; dies = day, or, "about a day long") to emphasize the fact that these rhythms actually differ in period from the dian twenty-four hour rhythm. In either circumstance, some kind of "biological clock" appears to be operating, "timing" these regularly recurrent behaviors.

How these "biologic clocks" are "driven" or "set" is not now completely understood and constitutes one of the central problems in biology today. Two alternative hypotheses have been offered to explain these rhythmic functions.

The first is that each organism is timed by an autonomous, endogenous, intrinsic, inherited and intracellular oscillator. This hypothesis postulates that each organism has within it its

own "clock" by which it "sets" its rhythmic activities, and that this evolutionarily related internal clock matches closely and adaptively the major geophysical periods.

The second hypothesis postulates that each organism's timing is dependent upon exogenous, extrinsic inputs from the pervasive, subtle, rhythmic, geophysical forces from which the organisms are never fully shielded. Such forces include the Earth's gravity, and its magnetic, electrostatic and electromagnetic fields.

Until recently it has been impossible to remove an organism of known biorhythm completely outside the Earth's rhythmic sphere of influence. Now with the opportunity to use space as a laboratory such removal becomes possible. The S061 experiment was based upon the need to deprive an organism of significant information about its rhythmic geophysical environment, and measure the effect of such deprivation upon the observed biologic rhythm. The potato, Solanum tuberosum, (whose dian oxidative metabolic rhythm seemed well-defined) was selected for a flight test to separate an organism from the Earth's geophysical influences. If the rhythm remained unaltered, the intrinsic hypothesis would appear more tenable; if the rhythm was altered, the extrinsic clock hypothesis would be supported. In short, any behavioral change would be informative, and provide insight into this crucial biologic question whose answer has many important basic and applied connotations.

2.2 Single Specimen Respirometer Development Review

The biological clock phenomenon discussed above were deemed by a scientific panel and NASA to be of sufficient importance to warrant eventual study in the space environment. Equipment available in the early 1960's for biorhythmic studies was unsuitable for space flight applications. As a result, Space/Defense Corporation contracted to develop a laboratory prototype capable of the pressure and temperature control and measurement required for space flight respirometric studies. Primary prototype considerations were: size, weight, power consumption, reliability and resolution.

2.2.1 Design

The principal design objectives were to design a life support system able to maintain a sprouting potato plug for 90 days in the space environment and measure oxygen consumption with accuracy and reliability. The plug, about 2.0 cm in diameter and 3.0 cm long, in darkness consumes oxygen and produces carbon dioxide with an R.Q. of about 1.0 at a rate of about .01 ml O_2/gm/hr. For the purposes of the design it was further assumed that this respirometer would be compatible with an unmanned spacecraft such as PIONEER.

The primary biologic data requirement was to resolve 2-3% variation in oxygen consumption, deviating from a mean basal rate of .06 ml O_2/hr for a 6.0-7.0 gm specimen. The basal rate range was assumed to be from .006 ml O_2/hr to 0.6 ml O_2/hr. (In actual practice, the oxidative metabolism requirement

of a 7.0 gm specimen varies from "zero" to, occasionally and briefly, about 1.2 ml/hr). Other limiting parameters were set at: weight about 5 lbs; volume, about 200 cubic inches; and power, about 100 milliamperes at 28 VDC (less data management power requirements and temperature control). The respirometric chamber was to: be in total darkness; control pressure at 760 mm Hg $\pm$ 2 mm Hg; maintain a nominal gaseous environment of about 20% oxygen, 80% nitrogen and the trace gases of air. Carbon dioxide was to be scrubbed to less than 1% and relative humidity was to be 90% or greater. Temperature was to be controlled at $68^{o}F \pm 0.5^{o}$ (by the space craft).

Preliminary design studies resulted in a configuration as shown in Figure 1. The differential pressure transducer approach was selected because no absolute pressure transducer meeting specifications were then available.

Continued analysis and a breadboard circuit showed that the transducer/control circuit subsystem would require discrimination of one part in 200,000 (5×10^{-6}) in the subcarrier oscillator output to discern variation in oxygen consumption. Due to drift and other instability problems we decided that this original metabolic measurement approach was probably not operationally feasible. Accordingly, several other alternative methods were examined and tested for suitability in the laboratory as set forth below.

Radioisotopic measurement, using Kr_{85} as a trace gas in the oxygen supply, was not suitable because of the inability to discriminate significant count differentials at the low oxygen consumption rates.

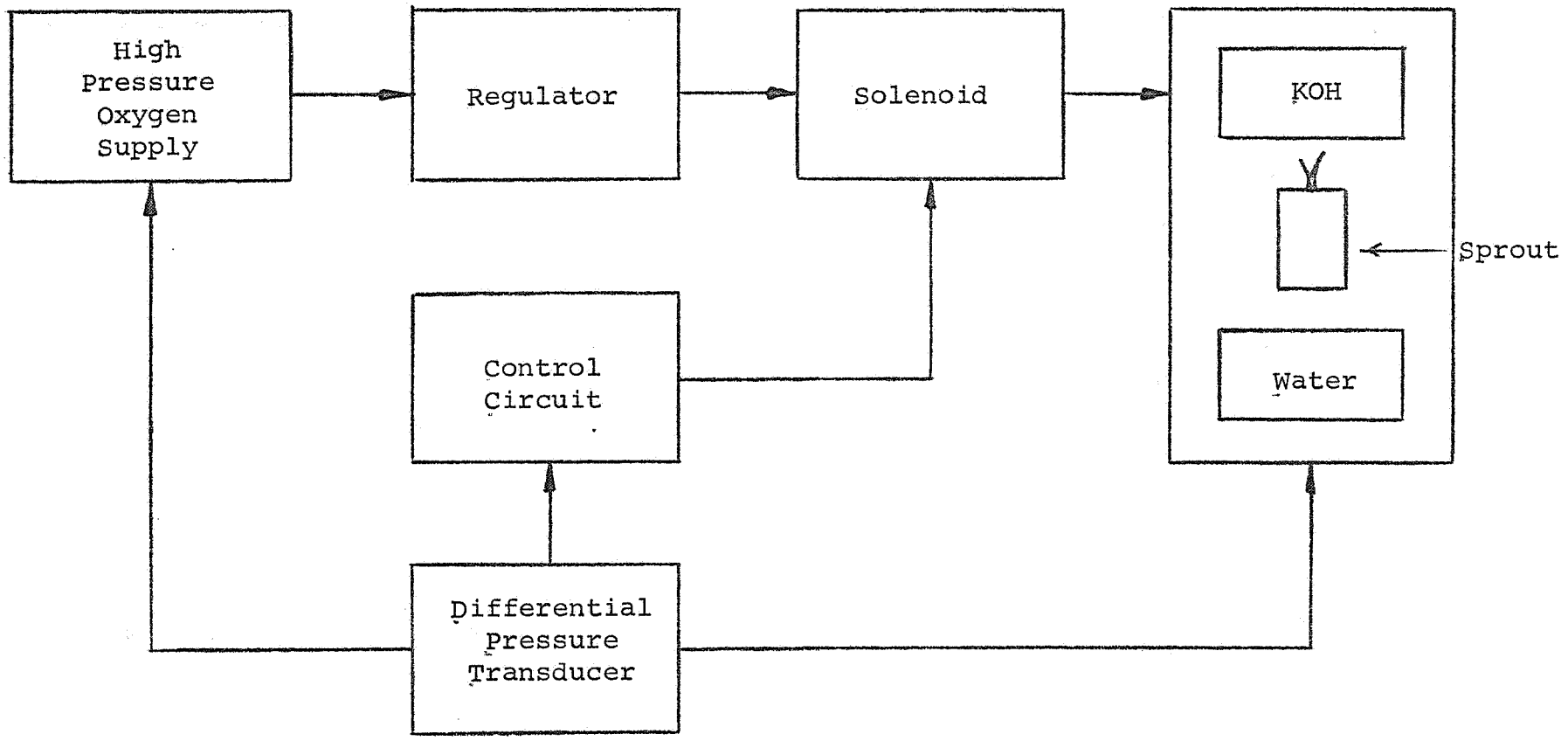

Figure 1.

Schematic of Initial Respirometer Configuration

Electrobiopotential measurements were attempted. The sprout's cellular biopotentials were measured but the records obtained did not correspond to known patterns of oxidative metabolism. Also, problems of injury potential and specimen-electrode interaction degraded the data quality and credibility.

Temperature measurements of the potato plug were made in an attempt to discern temperature variations as a function of oxygen consumption. Though the method showed promise, the penalties in equipment size, weight and power were intolerable.

Finally, a two-stage regulator configuration was devised. This two-stage system for supplying metabolic oxygen to the organism permitted an improvement of 5×10^2 in data resolution over that of the original single-stage system. Figure 2 schematically represents the respirometer employing the two-stage resupply technique. The operation of this system is as follows.

The first-stage bottle is charged with a sufficient supply of oxygen to provide for the metabolic needs of the organism for the 90 day duration of the investigative period. An integral pressure regulator controls the outlet pressure from this bottle at approximately 820 mm Hg absolute pressure. A solenoid valve in the supply line between the first-stage bottle and the second-stage bottle is provided to control the flow of oxygen between these stages. The second-stage bottle is identical to that of the first-stage with the exception of the charge

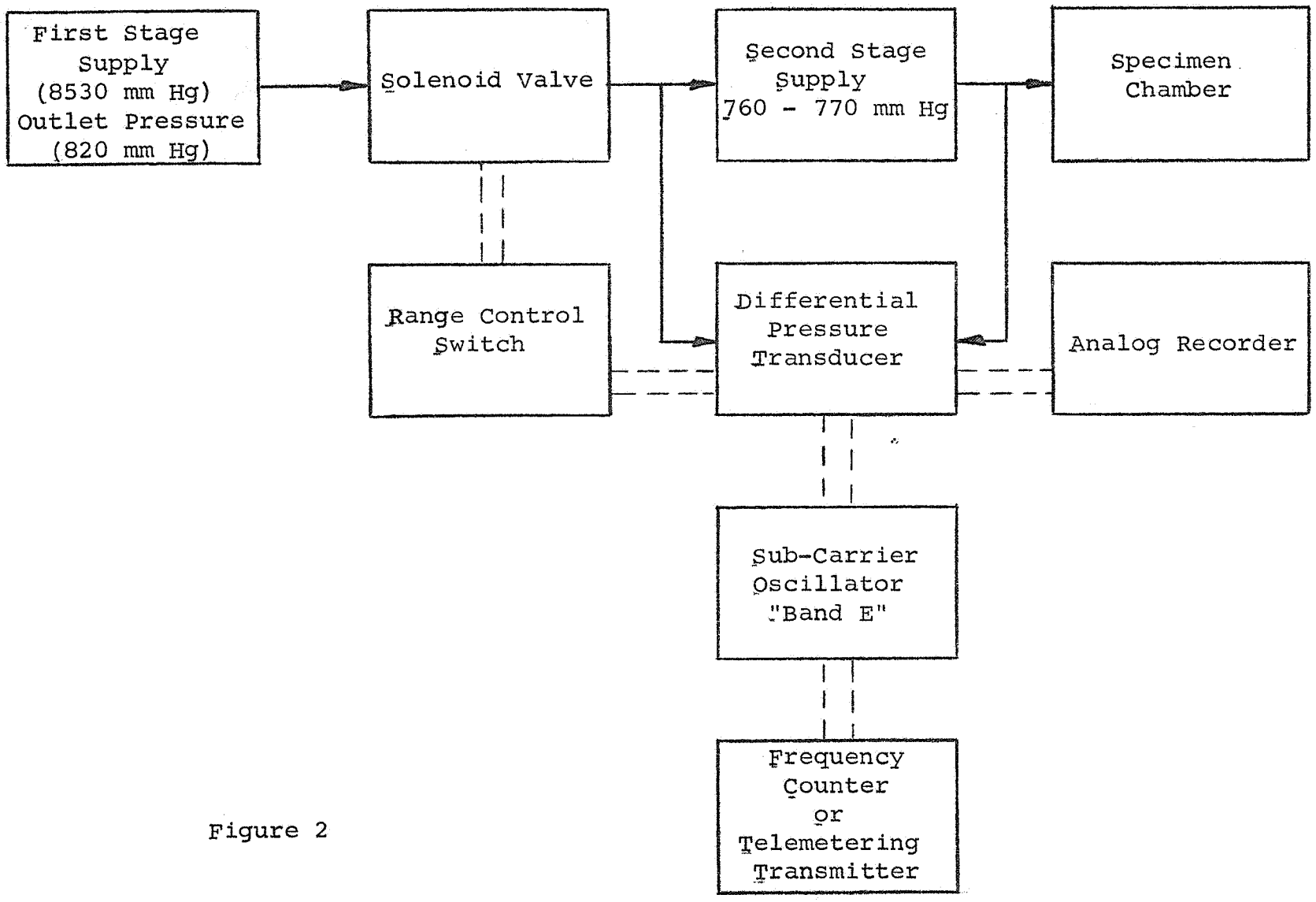

Figure 2

Schematic Diagram Two-Stage Resupply and Monitoring System

pressure and the regulated outlet pressure. The outlet pressure from the second-stage regulator is set to maintain a constant pressure of 770 mm Hg absolute in the specimen chamber. A differential pressure transducer which is connected between the specimen chamber and the second-stage supply bottle provides an output signal of 0-5.0 VDC for a differential pressure range of 0-0.3 psi. This output signal is the modulating signal for a voltage controlled sub-carrier oscillator having a double bandwidth of 20,000 cycles at a center frequency of 70 kHz. This 0-5.0 VDC signal is also utilized to trigger a specially developed solid-state switch for opening the solenoid valve when the pressure differential between the specimen chamber and the second-stage bottle approaches zero. The valve remains open, allowing oxygen to flow from the first-stage to the second-stage bottle, until a pressure differential of 0.3 psi (786 mm Hg abs.) has been re-established; at which time it is automatically closed.

The significance of this approach is readily apparent - it permits the monitoring of the rate of pressure change in the second stage bottle which may be correlated to oxygen consumption by the biological specimen in a zero leak system. This rate of pressure change is relatively rapid because of the small mass of oxygen (approximately 0.25 ml @ 1 Atmos STP) which must be metabolized to cause a full scale (0-5.0 VDC) change in the output signal from the differential pressure transducer. This rapidly changing signal, when plotted as a function of time, presents a line slope such that relatively small changes in consumption rate are indicated by significant changes in the slope of the differential pressure-time curve.

In the laboratory this information may be taken from the respirometer as either a digital or an analog output signal. In the former instance the output frequency may be read out by means of a frequency counter, and in the latter the output signal from the differential pressure transducer may be fed to a strip chart recorder or to a digital voltmeter for continuous monitoring of the rate of oxygen consumption.

2.2.2 Development and Test

One respirometer of this configuration was fabricated and placed in operation demonstrating both prototype function and problems. Subsequently three other devices were fabricated and all four placed on line with the purpose of collecting biologic data over a sufficient time period to demonstrate the suitability of the device to achieve experiment objectives. A number of major development problems (singly and in combination) occurred during the subsequent months. Typical of development problems, causes were not always easily related to effects; indeed, the observed discrepancy was often a manifestation two or three times removed from the primary problem. Furthermore, problems once thought to be "solved" reappeared in other guises, sometimes re-initiated by a "fix" to yet another problem. The sequence of events and their solution is important only in context of the epistemology of technologic development; nevertheless, the problems should be described.

Leakage. Internal leakage (usually interpretable as low oxygen consumption) and external leakage (usually

interpretable as high oxygen consumption) was an early problem. If both types of leakage were combined, the data was interpretable as either high or low consumption, depending upon the rate and rate of change of each leakage type. This difficulty was particularly puzzling at first, but as external leakage was controlled the "combined leak" interpretations were no longer of concern.

External leakage was reduced to a minimum by design, materials and handling techniques. Design approaches featured a minimum of external leak points combined with individual junction design features. "Edge-in-groove" seals (with an O-ring in the groove) were used as often as possible while other configurations (and particularly "edge-to-side" seals) were avoided. A variety of O-ring material were tried including malleable metal, organo-metallics, and natural and synthetic polymerics. A Neoprene varietal finally proved most satisfactory. Other anti-leak materials included the use of stainless steel (wherever possible) and hard aluminum alloys, surface-hardened after machining. Joints and junction seals were carefully greased and/or cemented, and re-sealing was kept to a minimum to reduce galling and spalling. By means of these design, material and handling precautions external leakage was controlled so that a "zero-leak" system evolved; i.e., with the instruments available, we were unable to detect the external leakage present.

Solenoid Valve. Commercially available solenoid valves proved unsatisfactory because of cost or unreliability,

often acting as regulators rather than valves, sticking open or shut and in general failing to meet the rigorous requirements of this system. Several design approaches were tested to determine the best configuration. A solenoid-driven belleville spring, seating and unseating a ball, was rejected because of an unacceptably wide dead-band in the spring's characteristics. A "magnet-loaded-shut" solenoid was also developed using a permanent magnet instead of the conventional spring. This proved unsatisfactory because of quality control problems in balancing each permanent magnet's force against each electro-magnet's force. Finally, a modified approach to the conventional solenoid configuration proved most successful. This utilized a short spindle with numerous turns generating one pound at zero gap. Higher forces were desired to provide better sealing but could not be generated without unacceptable weight, heat and power penalties. Despite this inadequacy, the device proved to be functionally satisfactory. This second generation valve is characterized by: fabrication from non-oxidative materials; external fine control of gas flow; elimination of potential leak points by plumbing redesign; and, finally, a more positive valve closure assuring no internal leakage. This solenoid routinely allows collection of about 45 - 50 days of continuous data with no solenoid dysfunction.

Regulators. After the initial problems associated with the anaeroid design and development had been resolved, it became clear that a major and persistent problem would be the hysteresic response characteristic of the regulators. This characteristic arose from the fact that the forces required to unseat the regulator valve, upon demand by the anaeroids, sometimes exceeded the forces available, because of the small pressure

differentials being sensed. This problem of inadequate force was not particularly bothersome in the first stage regulator where inlet and outlet pressure differences are small ($\leq$ 6 mm Hg). The amount of force that can be generated by a small anaeroid bellows across that pressure differential, coupled with the nearly balanced gas pressure on either side of the valve, barely serve to unseat valve especially when the valve must be essentially zero leak. The latter constraint implies either high seating forces or alternatively, extremely tight fitting valve faces. Since the force was not available, development efforts centered on excellent seal designs.

The then current state of the art in valve technology was a knife edge valve against a deformable seat. The design evolved into a circular knife edge against a ring seat, with the gas path as indicated in Figure 3. The dimensions involved are small: maximum diameter of the ring seat is 0.5 cm; the diameter of the hole, A, is .0635 cm. A major problem quickly was identified: if the force imbedding the circular knife edge into the ring seat was sufficient to assure zero leak, then the force needed to extract it was more than the anaeroid bellows could generate at the low pressure differentials encountered in the second stage regulator. The problem manifested itself in hysteresic regulator behavior; i.e., an undesirably low pressure had to develop in the low pressure side of the regulator before enough force was generated by the anaeroid bellows to extract the circular knife edge from the ring seat. The net result was to distort the resulting respirometric data, making it appear that the specimen was respiring at a slower rate than was, in fact, the case.

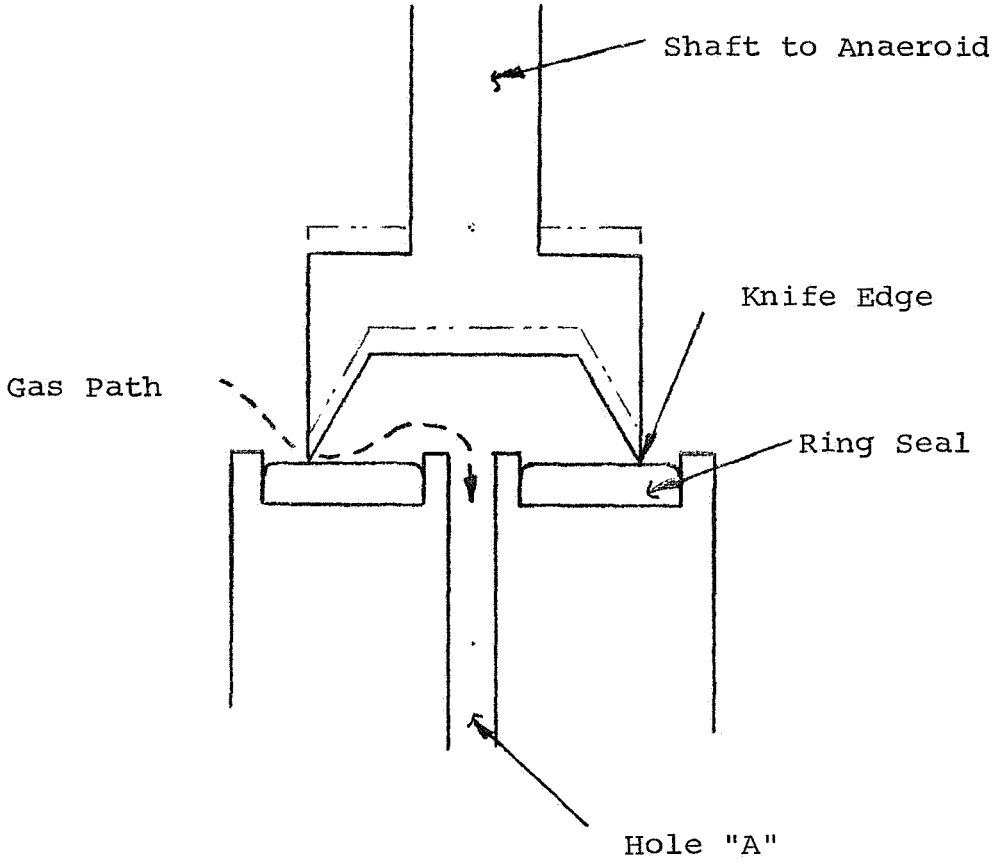

Figure 3.

Cross Section of Circular Knife Edge Valve Against Ring Seal

The solution to the problem, it seemed clear, was in selection of knife edge configurations, materials and surfaces and ring seat materials, optimizing for sealing efficiency as a function of sealing forces. The best combinations developed by late 1968 - early 1969 were not really satisfactory, requiring much "art" to produce acceptable results. Accordingly, in early 1969, a totally new approach was attempted using a push-rod-ball-bevel seat design.

The new concept proposed a ball and a lapped seat, both of sufficient hardness and smoothness to produce zero leak at low forces with low flows and high regulatory precision. Stainless steel, quartz, ruby and sapphire balls against a variety of metallic seats were tried, but without notable success because of deformation, spalling, dirt and the like.

We were forced to return to variations on the initial theme, concentrating on "semi-soft" seating materials and high quality control in preparation of the seat. Two currently acceptable approaches are outlined below.

One acceptable approach consists of the original circular knife edge of stainless steel, pressing against a ring seal made of Dow Corning 3116 silastic polymer which has only recently become available. The ring seal is prepared using the following technique: a lapped-smooth glass surface is pressed against an excess of 3116 poured into the ring seal groove. When the polymer has "set-up", the glass is removed, leaving

behind an acceptably smooth surface for engagement by the knife edge. Another successful variation of this technique is to flow a thin layer of 3116 into the ring seal groove. If done properly, a resilient, good seal develops which does not imbed the knife edge. Evidently, both of these variations require substantial "art," and so are (relatively) undesirable.

The second acceptable approach uses a sheet of natural latex 0.023 cm thick fastened to the top of the ring seal groove with Eastman 110 adhesive. After drying, excess latex is trimmed away and a hole about 0.005 cm is punched in the sheet using the gas passage as a guide for a sharply honed straight surgical needle.

2.3 Multispecimen Respirometer Development Review

Late in 1965, the Office of Space Sciences and Applications determined that the development of a multispecimen respirometer was desirable. The determination was based on biostatistical considerations. While weight, power and volume constraints, in some space flight situations, might decree that only one specimen could be flown, it is clear that credibility of experimental results are improved by larger samples, where spacecraft requirements are not so restrictive. Accordingly, the development of a multispecimen device was begun in early 1966, as outlined below.

2.3.1 Design

The design objectives were essentially identical to those established for the single specimen device as described in 2.2.1, above. The principal additional requirement was the need to support and measure a biostatistically significant sample. This requirement predicated an n of ten or more to allow at least nine degrees of freedom thus permitting use of the "small sample" biostatistical techniques of Yates, Fisher and others. To account for the possibility that one specimen might expire during the flight experiment and still leave an n of ten, eleven specimens were the minimum number needed. When contemplating a minimum number of eleven, practical layout considerations then dictated the use of twelve specimens, each in individual chambers arranged in two concentric rings of six each.*

The life support system for twelve specimens used multiples of the single cell technology; i.e., each cell was supplied from its own second stage regulator and oxygen store. These, in turn, were fed oxygen by a series of solenoids modulating gas flow from a single first stage regulator and primary oxygen supply. The need to supply and measure individual life cells required the design of a commutating valve. The problems presented by development and feasibility testing of this design are described below.

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*For illustration of the layout see Figure 2 of S/D Technical Report TR67-101 dated 8 February 1967, submitted earlier.

2.3.2 Development and Test

Most of the problems discussed in 2.2.2, above, apply also to the multispecimen configuration. Internal and external leakage, regulator hysteresis and solenoid problems were present during the development and test of the "multispud" system, but not with the frequency and severity encountered in the single cell system because of improved understanding based on the single cell experience. Two major problems were encountered, however, as outlined below.

Rotating Valve. The design of the valve was straightforward; only one real design concept recommended itself. The design was simple, requiring only the alignment of a series of "stationary" holes in the body of the valve with a "mobile" hole in the rotary section of the valve. But when the requirement for "zero leak" is imposed the potential problems became formidable. Accordingly, the design was optimized for zero leak deliberately accepting some penalties in weight, volume and power. After that decision the reduction to practice in development and test depended upon the identification of appropriate and sufficient technology. The small size of the holes (0.064 cm, $\pm$ .01) and the precise hole alignment required (0.5° $\pm$ 15 sec.) to assure zero leak required that class 1000 or better cleanliness be maintained at the valve face. If dirt did intrude high hardness valves at the faces were required to avoid "leaky" scratches. The zero leak requirement itself demanded excellent finishes. Eventually these requirements for finish and hardness resulted in the following use of high technology in valve configuration:

Valve Body:	Material:	661 T6 Aluminum, Hard anodized
	Flatness:	3.0 helium wavelengths
	Finish:	3.0 microns
Valve Rotor:	Material:	440 C Stainless Steel, Heat treated to 61-62 Range on the "C" Scale
	Flatness:	1.0 helium wavelength
	Finish:	0.5 microns

One other problem intruded; "grease" of any origin. Human skin oils, organic vehicles in lapping compounds, stopcock greases, joint sealants, etc., etc., in even minute amounts served to "hang" the rotary valve. This increased torques on the rotary drive motor resulting in overheating and stalling. Rigorous cleanliness using Class 1000 or better technique and water final wash essentially managed this problem.

2.4 Summary Evaluation

The development of the respirometer systems, on the whole, was successful. The effort resulted in the design, development and laboratory utilization of a new generation of micro-respirometers of unequalled sensitivity and accuracy (as a function of size, weight, volume and power) when compared to other respirometric devices. Their ability to support simple organisms for long periods (weeks/months) under rigorously controlled environmental conditions is also probably unequalled

even by laboratory devices much more complex, delicate and expensive (in both basic cost and operational cost).

The principal technologic inadequacy of the system is the (relative) unreliability of the second stage regulator and its reliance on "art" in the seals.

The principal program inadequacy was the (relative) cost-ineffectiveness of the effort. Because the contract support supplied each year was small (less than $40,000 per year) there was never enough money to attack the problems frontally and simultaneously using a systems engineering approach. Instead, a "partial solution" engineering approach was imposed which, in the long run, cost more time and more money.

In cost beneficial terms, however, the program was a success. The goal and most of the objectives were attained demonstrating the feasibility of such a device for space flight. The benefit derived was the technologic experential base (both positive and negative) which permitted the subsequent highly successful development (under another contract) of a space-flight-qualified microrespirometer for space biology experiments.

3.0 GRAVITY AND NEUROHYPOPHYSEAL SECRETORY ACTIVITY

3.1 Biological Review and Rationale*

The supraoptic and paraventricular nuclei of the hypothalamus are connected by a bundle of nonmyelinated nerve fibers to the neural lobe of the hypophysis. The function of this hypothalamo-hypophyseal system was discovered by Fisher and Ingram and confirmed by more recent studies showing that the posterior lobe hormones are synthesized in the supraoptic and paraventricular nuclei, or in the preoptic nuclei of lower vertebrates. The hormones are separately "packaged" in small secretory granules in association with a larger carrier protein molecule and migrate down nerve fibers, ultimately accumulating in the posterior lobe of the pituitary. This accumulation of granules in neurohypophyseal nerve endings is taken as evidence that neurosecretory material is transported distally by axoplasmic flow.

The mechanism(s) by which granule migration or axoplasmic flow occurs is not known and constitutes an important biologic question. Because of the anatomic relationship of the hypothalamus and hypophysis, it appears reasonable that gravity might well have a responsibility in the migration of these neurosecretory granules; if it does not, other mechanisms controlling flow must be responsible. There are arguments suggesting that the combined carrier

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*For a more complete discussion and bibliographic citation, see S/D Technical Proposals P69-171, dated 19 March 1969, and P70-129A, dated 18 September 1970, submitted earlier.

protein and hormone package may be just large enough to be affected by Newtonian forces rather than the Brownian dynamics, differential concentration or active transport mechanisms usually implicated in molecular transportation. At any rate, there is sufficient evidence to warrant an empiric test of the role of gravity.

Certainly, the pragmatic significance of the work is evident; weightlessness causes water loss and several mechanisms have been implicated. But, as categorically stated by Academician V. Parin in a recent article on life in orbital stations "...still we do not know why the organism is dehydrated in weightlessness." Parin implicates gravity and it is possible that the hypothalamus is involved. With deeper understanding of gravity's role in hypothalamic secretion, important benefits in aerospace and terrestrial medicine may be realized.

To determine the precise role gravity plays in neurosecretory granule migration we proposed that the physiology of the hypothalamo-hypophyseal system be studied under a variety of modes in the one-G environment. The test animal chosen for these studies was fresh water teleosts. Fishes have a distinct advantage over other vertebrates since their orientation to gravity can be altered with little difficulty, thereby allowing gravity to act on the hypothalamo-hypophyseal system in a variety of axes.

The organ of equilibrium in fishes is located in the pars superior and consists of the semicircular canals and their

ampullae and a sac-like vesicle, the utriculus. In bony fishes the utriculus contains an otolith called the lapillus. The lapillus rests horizontally on the hairs of the sensory cells of the crista utriculi and responds to the force of gravity, thereby stimulating the sensory cells of the cristae. This stimulation of hair cells works in conjunction with sensors in the lower portion of the retina to maintain balance. Thus, with both eyes and utriculi intact, light from above and gravitational force from below keep the fish oriented in an upright position.

If one utriculus is removed, or a strong beam of light is directed toward the fish at right angles, instead of from above, the fish can be made to lean to one side or the other. When a fish has its utriculi removed from both inner ears, it can be made to swim at a 90° angle to the normal gravitational field if illuminated from the side, or even upside down if lighted from below.

Utilizing these techniques, we have initiated a study to vary precisely the direction of gravitational force relative to the anatomic axis of the hypothalamo-hypophyseal system. Our intent is to assess the influence of altered gravitational orientations on neurosecretory granule regulation. The next section describes the work already completed.

3.2 Review of Research to Date

During early work in 1966-1967, the major efforts were: design of an environmental control system; development of surgical

procedures for bilaterally eliminating the utriculi in fish; and inducing voluntary re-orientation relative to the Earth's gravitational field with altered light cues.

An electronic method (based upon variable impedance) was designed which continuously assessed and recorded the position of the fish relative to gravity. This sensor was incorporated into the walls of a Plexiglass environmental chamber. Water continuously flowed through this chamber at a controlled rate producing slow swimming movements of the fish and consequent horizontal (G_x) orientation in the field. Light entered the chamber through a narrow translucent stripe along the long axis on the side of the otherwise opaque chamber. In our laboratory, fishes, surgically deprived of their inner ear mechanisms, oriented to this external light stimulus. By gradual rotation of the chamber and its translucent stripe the position of the fish relative to the G field was altered.

The removal of labrinthine structures is performed by making a small incision 3 mm behind each eye in fish, anesthetized with MS222 and carefully removing the vestibular structures with dissecting forceps. The entire procedure is done with the aid of a dissecting microscope. Within minutes the fish is swimming in a tumbling manner, completely disoriented. This behavior continues until the third day when the fish begins orienting toward the single bright light source illuminating the otherwise darkened aquarium. It is at this time the operated fish also begins eating and interacting with other fish in the aquarium.

All behavior appears normal except for the orientation of the operated fish. As the light source is incrementally moved around to the bottom of the aquarium, the operated fish again demonstrates disoriented swimming behavior, but soon "locks on" the light and swims inverted.

A photograph illustrating the behavior of an operated fish continuously orienting to a light source at the bottom of a standard aquarium can be found in TR69-103, dated 6 March 1969, submitted earlier. The labryinthectomized fish is at center; note the inverted position, relative to his unoperated fellows at upper left and upper right.

During the period 1967 - 1968 the re-orientation studies with fish continued, but the major effort was directed to the biochemical investigations needed to determine the presence of vasopressin in the neurohypophysis. The objectives were to: synthesize arginine-vasotocin (teleost vasopressin); develop antibodies to the synthesized hormone; tag the antibody with a fluorescing molecule; and, preliminarily, determine cross-reactivity of the antibody with the endogenous hormone.

The accurate bio-assay of arginine-vasotocin in tissue sections taken from the hypothalamus, infundibulum and neurohypophysis of fishes exposed to different gravitational orientations was necessary for the assessment of gravitational effects on neurosecretory regulation. There is no good procedure for the detection and assay of endogenous arginine-vasotocin prior to its release from the pituitary. Gormori's chrome hematoxylin

and other histologic stains lack specificity and cannot be used with confidence to selectively identify vasotocin neurosecretory granules. It was, therefore, proposed in the second phase that a new approach, utilizing fluorescent antibody techniques, be undertaken to assay arginine-vasotocin at its sites of synthesis, transport and storage.

The localization of an endogenous hormone by its specific antibody depends on the availability of this hormone to produce antibodies. Arginine-vasotocin, in teleost posterior pituitary extracts, had been synthesized by Katsoyannis and du Vigneaud but discussions with Professor du Vigneaud and Sandoz Pharmaceuticals revealed that this octapeptide was no longer available in a synthetic form. Purification of this hormone from fish pituitaries had been worked out, however, and was straightforward. We, therefore, proposed to purify arginine-vasotocin from crude pituitary extracts utilizing Rasmussen's techniques and use this material to produce antibodies for the fluorescent antibody assay of endogenous hormone in experimental and control fish. We soon discovered, however, that the amount of hormone necessary for antibody production proved to be in excess of that which could be reasonably purified. Consequently, we undertook the synthesis of arginine-vasotocin in our laboratory utilizing the Merrifield technique. By late 1968 we had successfully synthesized and purified two preparations of the active hormone for antibody production.

The antigenicity of vasopressin, oxytocin and arginine-vasotocin has been questionable due to their low molecular weights. Recently, however, Spragg, et al, successfully produced antibodies

to bradykinin (nine amino acids) through both coupling and polymerization techniques. We utilized Freund's adjuvant and employed similar and polymerization methods to determine the best procedure for making antibodies to arginine-vasotocin. These antibodies were, in turn, used to detect endogenous vasopressin by labeling the molecules with a fluorescent tag and selectively staining sections of hypothalamus, infundibulum and pituitary. Rabbits were innoculated with synthetic arginine-vasotocin and antibody activity was determined periodically, using the ring test. Titers were pooled and the globulin fractions purification was attempted, using the Ethodin (Rivanol) procedure described by Horejsi and Semtana.

Anti-arginine vasotocin was coupled with Rhodamine B-200 (a fluorescent tag) and the conjugated globulin was chromatographed on a Sephadex G-25 column with phosphate buffered saline (pH 7.2) for purification. The conjugated globulin was subsequently stored in a frozen state until used to identify endogenous arginine-vasotocin in the hypothalamo-neurohypophyseal system of angel fish (Petrophyllum scalare). But because of low yields of our synthetic arginine-vasotocin, we also made antibodies in goats to commercially available oxytocin in the belief that these antibodies would cross-react with endogenous fish arginine-vasotocin thereby enhancing our fluorescent antibody staining capability. We tagged these antibodies and determined their cross reactivity.

In addition to the above, we established a large (530 gallon) holding facility to house fish at controlled environmental

conditions thereby enabling us to grow healthy fish to a size that will minimize surgical and pituitary sectioning problems.

Difficulty of data interpretation from the variable impedance position detection device forced development of a more reliable method.

Continuous surveillance (24 hours/day) of experimental and control fishes was achieved with time lapse photography and enabled us to assess the success of surgical procedures on our experimental and sham-operated animals.

A combination control and experimental all-glass aquarium has been constructed with a variable light source that can be altered to produce the desired orientation of labyrinthectimized fishes and provide adequate light for time-lapse pictures. It also produces a variable rheotropic environment.

3.3 Summary Evaluation

The research to date, has been fairly cost-effective considering that the work is quite basic. Few "dead ends" have been encountered either in hardware development or in biological aspects. The principal disappointments have been in development of a reliable fish orientation detector not dependent on human data analysis. All the systems developed to date (except photographic) cannot differentiate between an "upright" or an "inverted" fish, though we can detect a fish swimming at $90^{o} \pm 15^{o}$ to normal. Another disappointment was the failure to generate adequate titers

of antibody in rabbits to the synthetic octapeptide. Otherwise, all major goals and objectives have been attained to date. In addition, serendipitous advantages (making the research cost beneficial, in our opinion) have been realized. The application of solid state (Merrifield) techniques to successful synthesis of an important hormone was an unexpected benefit.

The biochemistry and immunology in this program have been quite sophisticated and we are professionally gratified by our developing skills at the forefront of this science and technology. But, candidly, our achievements are not more impressive than those of our peers, and have been sufficient to attain the program objectives. New skills will be needed to prosecute the work now underway (under a new contract, NASw-2160) and are briefly outlined below.

3.4 Future Work

Future efforts will be devoted to the collection and analysis of data from labyrinthectimized and control fishes. Labyrinthectimized fishes will be subjected to altered gravitational orientations for various periods of time and their neurosecretory physiology will be compared with control fishes subjected to identical environmental conditions. All data will be statistically analyzed.

The following experimental design is contemplated. Angel fish, and/or goldfish acclimated to our laboratory conditions will be used for experimental and control animals. Four

groups of animals will be bi-laterally labyrinthectimized. One control group will be sham-operated. One un-operated group will serve as baseline control. The operated fish will be induced to swim at different angles relative to the normal gravitational force field for varying periods of time.

Sham-operated and un-operated control fish will be allowed to swim within the same system, but separated from experimental fish for the same time periods (i.e., 1, 2, 4, 6 and 8 weeks). Time lapse pictures (one frame/two minutes) will be taken 24 hours/day throughout the entire experiment to confirm orientation positions along with the behavioral and physiological status of all animals.

After operated and control fish have been subjected to specific gravitational orientations for the desired period of time, they will be sacrificed and their brains serially sectioned for staining with fluorescent antibody solutions.

Controls for establishing the specificity of staining will be utilized. The globulin fraction of pooled, normal rabbit serum will be conjugated to Rhodamin B-200 and purified in the same manner as the arginine-vasotocin antibody. This labeled fraction will then be used as a control for the staining procedure. As an additional control procedure, fluorescent antibody binding will be blocked by pre-incubation of a section with unconjugated antibody and compared with fluorescently-stained sections.

To supplement the above fluorescent antibody assay technique, we will develop and employ a bioassay procedure for kidney based on adenylcyclase activity that we believe will add greatly to endocrine studies of the hypothalamo-neurohypophyseal system and increase significantly the meaningfulness of our present study.

The adenylcyclase bioassay, coupled with our fluorescent antibody assay technique, will provide quantitative information on the presence and biological activity of arginine-vasotocin in various portions of the hypothalamo-neurohypophyseal system and circulatory system. This data will be correlated with gravitational influences if gravity does play a role in its regulation